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"Meiotic genes" are constitutively expressed in an asexual amoeba and are not necessarily involved in sexual reproduction

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Abstract

The amoebae (and many other protists) have traditionally been considered as asexual organisms but suspicion has been growing that these organisms are cryptically sexual or are at least related to sexual lineages. This contention is mainly based on genome studies in which the presence of "meiotic genes" has been discovered. Using RNA-seq (next generation shotgun sequencing, identifying and quantifying the RNA species in a sample), we have found that the entire repertoire of meiotic genes is expressed in exponentially growing *Acanthamoeba* and we argue that these so called meiotic genes are involved in the related process of homologous recombination in this amoeba. We contend that they are only involved in meiosis in other organisms that indulge in sexual reproduction and that homologous recombination is important in asexual protists as a guard against the accumulation of mutations. We also suggest that asexual reproduction is the ancestral state.

1. Introduction

It is currently assumed that sexual reproduction is the ancestral state of eukaryotes and that asexuality arose later [1-4]. Meiosis is necessary in sexual reproduction to produce haploid gamete cells. These gametes then fuse to form a fertilized egg in which parental genomes rearrange to produce a unique diploid nucleus. This being the case, an organism cannot reproduce sexually without genes that facilitate meiosis, but some have inverted this argument and suggest that the possession of meiotic genes indicates a facility for sex [5]. The list of protists in which these meiotic genes have been discovered and for which sexual reproduction has therefore been inferred is growing, and presently includes *Entamoeba, Leishmania* and *Giardia* [6], *Ostreococcus* [7], *Trichomonas* [8], the choanoflagellate *Monosiga* [9], algae [10], mycorrhizal fungi [11], the dinoflagellates [12,13], the freshwater amoeba *Cochliopodium* [14], and the soil amoeba, *Acanthamoeba* [15]. Evidence does exist for sexual processes in a minority of these groups such as *Leishmania* [16] but there is no evidence for this in others such as *Acanthamoeba*. It has also been pointed out that these "meiosis genes" may have other functions such as homologous recombination [17,12] in polyploid organisms including *Acanthamoeba* [18]. Here we report that all genes previously classified as being meiosis specific are expressed constitutively in exponentially growing *Acanthamoeba* cultures in which no cell fusion has been reported. We therefore conclude that they are not likely to be involved primarily in meiosis and speculate that in *Acanthamoeba* they have other functions such as homologous recombination (the exchange of genetic information between two extensively homologous strands of DNA).

2. Materials and Methods

Two strains of *Acanthamoeba* (GS-336 and SB-53) were used in this study both of which are of the T4 genotype and both are closely related to the Neff strain (ATCC 30010) for which a complete genome is available [19]. *Acanthamoeba* strains were grown in axenic media (Bacto tryptone 14.3 g/L, yeast extract 7.15 g/L, glucose 15.4 g/L, Na_2HPO_4 0.51 g/L and KH_2PO_4 0.486 g/L pH6.5) in which the doubling time was measured to be 8.5 hours at 20^{o}C. RNA was extracted from exponential *Acanthamoeba* cultures using an RNeasy Mini Kit (Qiagen). The quality of the RNA was determined by agarose gels and by a QUBIT RNA BR (broad-range) Assay Kit (Thermo-Fisher Scientific). cDNA Libraries were prepared for automated TruSeq stranded mRNA-seq from the total RNA from single culture of the two *Acanthamoeba* strains. The sequencing data generation was made with HiSeq-4000 75PE by Edinburgh Genomics. The reference genome (FASTA and GTF files) from *Acanthamoeba castellanii* was obtained from ENSEMBL Protists [19,20]. Raw data quality control was performed using the FASTQC program (Simon Andrews https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The genome was indexed and reads

were aligned to the reference genome using STAR to obtain the required BAM files [21]. The alignments and the BAM files were visualised using SAMtools and IGV to verify the quality of the results [22,23]. GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and amoebaDB (http://amoebadb.org/amoeba/) were searched for meiosis specific genes.

A complete set of "meiosis genes" has been identified in *Acanthamoeba* through GenBank searches and by BLAST searches using known homologs from a variety of other organisms. The identification of each candidate has been studied by phylogenetic analysis to ensure that the *Acanthamoeba* homolog position was compatible with isoforms from other organisms. Where there was more than one candidate gene, phylogenetic analysis and direct pairwise sequence comparisons of better characterised orthologs from other species were made to ensure that the correct *Acanthamoeba* ortholog had been selected. Sequences were compiled using Seaview [24] and BioEdit [25] was used to edit alignments by eye and to determine levels of identity. Maximum likelihood phylogenetic trees were created with PhyML [26] using the GTR model with 100 bootstrap pseudo-replicates.

3. Results

We have analysed a set of meiosis-specific genes by maximum likelihood phylogenetic analysis to ensure that these genes are likely to be homologs of meiosis specific genes identified and characterised in other organisms. An example (Hop2) is shown in Figure 1 where the identified *Acanthamoeba* homolog branches in the expected position with good support among the amoebozoa. All other meiosis-specific *Acanthamoeba* homolog genes have been similarly tested. Only two meiosis-specific genes within the set studied here were found to have more than one candidate in the *Acanthamoeba* genome. In both cases it was clear from the phylogenetic tree analysis and by individual pairwise sequence comparisons which of these was the best candidate for the *Acanthamoeba* homolog and these were selected for this study.

The specificity of the RNA-seq approach was tested by searching for cyst specific protein genes as a negative control ACA1_075210, ACA1_075240, ACA1_ 327930, ACA1_399800 and none of these appeared in our expressed protein data base. Cyst specific protein 1 is expressed in *Acanthamoeba* as it differentiates into cysts [27]. As expected, actin (ACA1_361250, ACA1_361250) and EF1α (ACA1_138040) genes were heavily expressed (Figure 2). The lack of cyst specific transcripts confirms that these particular cultures are in log phase as cysts form in post-log phase.

We have discovered that all the identified meiosis genes are expressed in exponentially growing amoebae indicating that the expression is not restricted to cells undergoing meiosis (Figure 2). These genes include the core genes (Spo11, Hop1, Hop2, Mnd1, Mlh1, Mlh2, Pms1, Dmc1, Msh2, Msh4, Msh5, Msh6, Rad50, Rad51, Rad52) that are ''meiosis-specific'' since they are known to orchestrate meiosis only in organisms with a sexual ancestry [6, 8, 15]. Two other genes, HAP2 and GEX1, have been included in the present study as they are involved in cell and nuclear fusion and so have been used as markers for sexual reproduction [2].

4. Discussion

Current opinion tends to consider sexual reproduction as being ancestral and that asexual organisms have subsequently lost this ability [1,2]. On theoretical grounds it has been concluded that asexual reproduction can only be transient as such organisms would experience the accumulation of deleterious mutations. This has become known as Muller's ratchet [28]. However, a counter to this argument is that Muller's ratchet does not operate in organisms that are polyploid as the productive mutation rate is limited by correction through homologous recombination [18]. It has been argued that the bdelloid rotifers have adopted another way around the problem of Muller's ratchet without sexual reproduction through extensive horizontal gene transfer [29]. However, this idea has been

challenged by the observation that the genomic DNA used for this study was significantly contaminated [30]. It is interesting to note that like *Acanthamoeba*, the genome of the bdelloid rotifer *Adineta vaga* contains a set of core meiotic genes in the clear absence of meiosis [31].

In some lineages that have been viewed as being asexual, evidence has been discovered for the existence of sexual reproduction. The general trend is for members of the excavata, sexual reproduction tends to dominate. This has been described in *Trypanosoma* where cell fusion is reported [32], in *Naegleria lovaniensis* inferred from isoenzyme analysis [33] in microscopic analysis of *Leishmania* amastigotes within macrophages [16], from population genetic analysis in *Giardia* [34], and in *Trichomonas* [35]. The amoebozoa seem to be dominated by asexual members such as *Entamoeba* and *Acanthamoeba*, but meiosis and sexual reproduction has been demonstrated in others, for example by genetical analysis in *Dictyostelium* [36], and by morphological examination in *Cochliopodium* [4, 37] and in the testate amoeba *Arcella* [38]. Many protists including those assumed to be from the most primitive lineages show no indication of sexual reproduction. A growing list of organisms that were assumed to be asexual but which are found to possess meiosis specific genes are suspected to have a sexual reproductive capacity which may be hidden by culture conditions. For example, Ramesh and co-workers contend that "The presence of these genes indicates that: (1) *Giardia* is capable of meiosis and, thus, sexual reproduction" [6]. However, in our view, all that the presence of these genes indicates is that the lack of sexual reproduction in these organisms cannot be blamed on a lack of these genes.

The fact that all the meiotic genes are expressed in logarithmically growing *Acanthamoeba* in significant quantities means that they are unlikely to be primarily involved in meiosis since there is no indication that these amoebae are fusing or any other sign of meiosis or sexual reproduction. Although the difference between sexual and asexual reproduction is usually quite distinct, several redefinitions of the processes have lessened the distinction. True sexual reproduction usually includes meiosis to produce haploid gametes, cell fusion, then nuclear fusion, to form a diploid cell. Within the context of *Giardia*, 'sexual reproduction' and 'sex' have been defined much more broadly as "any process in which chromosomes from two cells, or two nuclei in the same cell, are combined in the same nucleus and undergo recombination to produce new genotypes" [40]. If we further broaden this definition to include the combination of two genes in the same nucleus, then gene conversion or homologous recombination can also be defined as 'sex'. This definition is unlikely to attract support, but it can be argued that traditional sexual reproduction and homologous recombination are at opposite ends of the same spectrum. It is our opinion however, that *Acanthamoeba* and similar organisms are best described as reproducing asexually and that the homologous recombination that is expected to operate between similar chromosomes in the polyploid nucleus cannot be described as sexual or even parasexual.

In summary, we argue that the presence of meiotic genes does not necessarily mean that meiosis is occurring as a prelude to sexual reproduction. We further argue that these genes are instead involved in homologous recombination between multiple copies of genomic elements in the polyploid nucleus of *Acanthamoeba* thus allowing this asexually reproducing amoebae to avoid the deleterious accumulation of mutations. Others too have suggested that meiotic genes have other functions [12] including homologous recombination [17, 40]. The same is likely to hold for some of the many other protists such as *Acanthamoeba*, in which meiotic genes have been discovered [15] but for which there is no other evidence for sexual reproduction. If this is the case then it makes it more likely that the theoretical last common eukaryotic ancestor was asexual. This would remove the awkward necessity of finding a compatible and compliant mate in the vast empty spaces likely to have existed at the time that these early cells lived. Sex is a very expensive and complex

phenomenon that is expected to have arisen well after these initially asexual populations, using the same set of genes used in homologous recombination.

Research ethics

No ethical consent was sought from the local ethics committee since it was clear that this was not necessary in this case.

Animal ethics

Only amoebae were used in this study and as these are neither sentient or conscious, ethical considerations are not applicable.

Permission to carry out fieldwork

No fieldwork was involved in this study.

Data accessibility

All sequence data involved in this study are accessible either through GenBank (https://www.ncbvi.nlm.nih.gov/genbank/) or the amoebaDB data base (http://amoebadb.org/amoeba/), and in most cases both. Sequence alignment data for figure 1 are available from the Dryad Digital Repository [41].

Author's contributions. S.K.M. conceived the study analysed the data and wrote the paper. A.O.F.V. and Z.K. isolated strains, performed the RNA-seq experiments, analysed the data, contributed intellectually to the paper's content and edited the manuscript. All authors have read and approved the final published version of this manuscript. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved

Competing interests. All authors declare that there are no competing interests.

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Figure/table legends

Table 1. Meiosis/recombination associated genes in *Acanthamoeba* and their expression level as determined by RNA-seq. *Acanthamoeba* homologs were identified by BLAST and confirmed by phylogenetic analysis. *LogCPM values reflect the level of expression of these transcripts in exponentially growing axenic *Acanthamoeba* cultures. The two values are derived from two separate measurements from two different *Acanthamoeba* strains upper value from SB-53, lower GS-336.

Figure 1. An unrooted phylogenetic analysis of Hop2 showing that the *Acanthamoeba* gene groups with the amoebozoa (orange group) as expected. Maximum likelihood analysis of the protein sequences showing branch support. The tree was created with PhyML [26] using the GTR model with 100 bootstrap pseudo-replicates.

Figure 2. The approximately 13,000 RNA transcripts are displayed in order of their relative abundance (blue bars) present in exponential (GS-336) *Acanthamoeba* cultures (SB-53 gave similar results). Most abundant transcripts left, least right. The "meiosis specific" transcripts are highlighted in red. The actin genes (ACA1_361250, ACA1_361250) and EF1α (ACA1_138040) show the highest expression.

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	Rad50	Rad51	Rad52	Spo11	Hop1	Hop2	Mre11	Mnd1	Dmc1
GenBank	XP_004339639	ELR18834	XP_004337923	ELR12359	XP_004340201	XP_004334651	ELR17651	XP_004340260	XP_004353078
AmoebDB	ACA1_063900	ACA1_166930	ACA1_188580	ACA1_374260	ACA1_369130	ACA1_091480	ACA1_064360	ACA_36980	ACA1_071720
LogCPM*	5.80 6.08	4.05 4.17	4.62 4.76	1.72 1.77	0.82 1.06	5.68 4.83	5.08 5.18	4.45 4.59	-0.05 0.11

	Pms1	Mlh1	Mlh2/Mlh3	Msh2	Msh4	Msh5	Msh6	HAP2	GEX1
GenBank	XP_004342239	XP_004351570	XP_004367469	XP_004337972	XP_004352766	ACA13171(part)	ELR15471	XP_004341525	XP_004341936
AmoebaDB	ACA1_115690	ACA1_149810	ACA1_195260	ACA1_031570	ACA1_068220	ACA_094390	ACA1_340910	ACA1_266960	ACA1_133490
LogCPM*	4.70 4.48	3.96 4.57	2.12 2.94	6.04 5.54	1.75 2.66	3.52 4.63	6.57 6.70	3.88 4.15	1.58 2.89

Table 1